Maintenance of primary cell cultures of immunocytes from Cacopsylla spp. psyllids: a new in vitro tool for the study of crop pest insects

UNIVERSITÀ DEGLI STUDI DI TORINO

Maintenance of primary cell cultures of immunocytes from *Cacopsylla* spp. psyllids: a new *in vitro* tool for the study of crop pest insects

Monti M.[1], Mandrioli M.[2], Bextine B.[3], Hunter W.B.[4], Alma A.[1], Tedeschi R.[1]

[1]Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy, [2]Dipartimento di Scienze della Vita, Università degli Studi di Modena e Reggio Emilia, Modena 41125, Italy, 3 University of Texas at Tyler, 3900 University Blvd., Tyler, TX 75799, USA, [4]USDA, ARS, U.S. Horticultural Research Laboratory 2001 South Rock Road, Fort Pierce, FL 34945, USA.

Corresponding Author:

Rosemarie Tedeschi

Dipartimento di Scienze Agrarie, Forestali e Alimentari (DISAFA), University of Torino, Largo P. Braccini 2, 10095 Grugliasco (TO), Italy.

E-mail: rosemarie.tedeschi@unito.it

Running title: Immunocyte cell cultures from *Cacopsylla* spp.

Summary

Primary cell cultures of immunocytes have been developed from the three psyllid species *Cacopsylla melanoneura*, *Cacopsylla pyri* (vectors of '*Candidatus* Phytoplasma mali' and '*Candidatus* Phytoplasma pyri', respectively) and *Cacopsylla crataegi*. The medium most suitable of those evaluated was HH70 psyllid medium. In fact, good survival and proliferation of the *Cacopsylla* immunocytes for over 60 days were observed, with mitosis activities starting at 15 days post culture. Moreover, adhesion and phagocytosis activities were confirmed for all the psyllid cell cultures by functionality tests. Morphological examination of cultured immunocytes revealed the presence of different cell types in all the three psyllid species in accordance to published data about insect immunocytes.

The *in vitro* maintenance of psyllid immunocytes represents a powerful tool for a wide range of applications, especially for psyllid cell biology. In particular, in depth studies on the biology of psyllids as vector insects as well as analyses to understand the mechanisms behind the interactions with pathogens and symbionts are now possible. These cultures can be used as an *in vitro* model to study psyllid humoral immune responses, which also will allow in-depth investigations on the abilities of psyllids as vectors of phytoplasmas. All these applications provide new opportunities to develop more focused and specific pest control strategies.

Key words: *Cacopsylla*, *in vitro* culture, immunocytes, adhesion, phagocytosis

Introduction

Several psyllids are known vectors of plant pathogens (Phytoplasmas and Liberibacters) and as such are regarded as economically important pests (Hodkinson et al. 2009). The availability of psyllid cell cultures will provide a greatly needed resource tool to evaluate and understand the interaction of pathogens at the cellular level and will open new opportunities for expanding management strategies against these devastating pests and diseases.

The genus *Cacopsylla* includes the most important vectors of fruit tree phytoplasmas as well as the vector of '*Candidatus* Liberibacter europaeus' (Carraro et al. 1998; 2001; Tedeschi and Alma 2004; Jarausch et al. 2007; Raddadi et al. 2011).

Building upon the success of Marutani-Hert et al. (2009) with the Asian citrus psyllid, *Diaphorina citri* (Kuwayama) mature embryos, this work focused on developing primary cell cultures from three psyllid species, *Cacopsylla melanoneura* (Förster), *Cacopsylla pyri* (L.), and *Cacopsylla crataegi* (Schrank). The phytoplasmas transmitted by *C. melanoneura* and *C. pyri* cause severe disorders to apple and pear trees, with important losses in terms of quality and quantity of the production. On the contrary, the role of *C. crataegi* in transmitting phytoplasmas has never been proven, despite its possibility of harbouring them (Tedeschi et al. 2008). In particular, we focused on immunocytes since previous investigations suggested that the host's cellular innate immune system mediates the host tolerance to symbionts and pathogens placing circulating immunocytes in a pivotal role (Su et al. 2013).

Insects and primary cell cultures

Adults of *C. pyri* (collected on pear trees), *C. melanoneura* (collected on apple trees) and *C. crataegi* (collected on hawthorn plants) in Northwestern Italy, were used to establish psyllid cell cultures. Insects were washed in 0.115% sodium hypochlorite, 75% ethanol and MilliQ sterile water for 10, 30 and 20 seconds (sec) respectively. After drying on a filter paper for a couple of seconds, they were put in a single well of a sterile 24-well cell culture plate (Costar®, Corning, NY, USA) containing 1 ml of Hert-Hunter 70 (HH70) medium (Marutani-Hert et al. 2009). The abdomen was cut apart and gently shaken with a pair of sterile forceps for 5-10 seconds in order to release the immunocytes, paying attention to avoid tissue ruptures. Then, together with the other solid parts of the insect, the abdomen was removed in order to avoid

82 migration into the medium of different cell types from the remaining tissues. To avoid dehydration of the cell
83 culture, 1 ml of sterile MilliQ water was used to fill adjacent wells. Two female adults were used for each
84 species for each well. Plates were incubated at 24-26°C and 0.2ml of medium was added every 48h if
85 necessary, whereas observation of cell cultures and the evaluation of the cell growth was carried out daily
86 using an inverted Leica DMI3000 light microscope.
87
88 *Media and supplements*
89 In order to assess the best growth conditions for *C. pyri, C. melanoneura* and *C. crataegi* immunocyte
90 cultures, three media were evaluated: Ex-Cell ® 405 (Sigma) and Sf-900™III SFM (Invitrogen, Carlsbad,
91 CA) with addition of 10 ml/L L-Glutamine200mM solution (Invitrogen) and the psyllid medium HH70
92 (Marutani-Hert et al. 2009). Each medium received antibiotics Gentamicin (at a final concentration of 50
93 µg/ml, Sigma-Aldrich, MO, USA) and Penicillin/Streptomycin (Sigma-Aldrich, MO, USA) at a final
94 concentration of 50U/ml and 50 µg/ml, respectively. The antimycotic agent Nystatin (Sigma-Aldrich, MO,
95 USA) was also added to each medium at a final concentration of 100 U/ml. Cell counts were performed with
96 a Bürker chamber (Brand GmBH, Wertheim, Germany).
97
98 *Cell morphological characterization*
99 An aliquot of cells (in a volume ranging from 40 to 120 µl on the basis of the cell culture density) was
100 cytocentrifuged onto slides with a Shandon Instrument Cytospin II running at 400 rpm for 2 min, then
101 stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence
102 microscope. Images were taken using a CCD camera (Spot, Digital Instrument, Madison, USA) and the Spot
103 software supplied with the camera and processed using Adobe Photoshop (Adobe Systems, Mountain View,
104 CA).
105
106 *Functional assays by adhesion test and phagocytosis assay*
107 An aliquot of 200 µl from each immunocyte cell culture was collected and placed on a glass slide in an
108 aseptical Lab-Tek Chamber Slide system (Nunc, Naperville, IL, USA). Immunocytes were allowed to attach
109 for 30 min in presence of HH70 medium. Thereafter, the slide was removed from the chamber slide system,

stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope. Photographs were taken using a CCD camera as previously reported.

For each cell culture a phagocytosis assay was performed. Briefly, a 200 µl aliquot was sampled and added to 100 µl HH70 medium in a 0.2 ml tube previously covered and the material was then incubated with 0.1 µl of a fluorescent beads suspension for 30 min in soft oscillation, according to Manfredini et al. (2008). After incubation, cells were cytocentrifuged onto glass slides, stained with a 200 ng/ml propidium iodide solution and observed with a Zeiss Axioplan epifluorescence microscope.

Results and Discussion

Maintenance of psyllid immunocytes in culture medium gave different results in the three different media evaluated (Fig. 1). The Sf-900™III medium did not support psyllid cells which shrivelled and died in the first two days post culture. Better results were obtained with the Ex-Cell ® 405 medium, since cells remained viable for more than one month even though with a low growth rate. On the contrary, extremely positive results were obtained with HH70 medium, which kept cells alive for more than sixty days. Cell counts showed a slightly declining cell number in the first 15 days with mitosis observed in cells cultured from the three psyllid species starting at 15 days post culture (Figs. 2, o-q) with a maximum amount of $3x10^4$ cell/ml at day 45. In all three *Cacopsylla* species different cell types were observed (Fig. 2), in accordance to published data (Lavine and Strand 2002; Pandey and Tiwari 2012). Most of the observed cells in the three studied species were small in size with the nucleus occupying the central part of the cellular body and they resembled typical insect plasmatocytes (Figs. 2b, c, f, g, l, m). A second type observed in all three studied species (Figs. 2a, c, e, h, i, n) consisted of cells larger than the previous with abundant cytoplasm containing cytoplasmic inclusions varying in shape from round to irregular or elongated. The third cell type (observed in *C. crataegi* only) (Figs. 2d, g) consisted of small cells with a thin cytoplasm layer without granules.

The presence of three different cell types and their shape and presence/absence of cytoplasmic inclusions is in agreement with previous studies indicating that prohemocytes, plasmatocytes and granulocytes represent the typical immunocyte types observed in insects (e.g. Manfredini et al. 2008). Adhesion tests showed that more than 80% of the psyllid immunocytes were able to adhere to a glass slide after 30 min

incubation (Figs 3a-c), losing their spherical shape. Moreover, more than 75% of the cultured cells were able to phagocytize fluorescent microspheres (Figs. 3d-g) indicating that they are functional despite their *in vitro* maintenance. These results are in agreement with published data suggesting that most part of immunocyte is able to adheres to the glass and to phagocytize fluorescent microspheres (e.g. Manfredini et al. 2008).

The application of immunocyte cell cultures in insect science has been widely increased in the last decades. In particular most of the interest has been focused on mechanisms underlying the cellular and humoral immunity and the interaction with microorganisms (Fallon and Sun 2001; Smagghe 2009).

The *in vitro* maintenance of *Cacopsylla* spp. immunocytes increases the opportunity for studying insect humoral immune responses, pathogen/host interactions, and host hemolymph microbe fauna. Evaluation of all three species of *Cacopsylla*, demonstrated that they can carry phytoplasmas and two of them are acknowledged vectors (Carraro et al. 1998; Tedeschi and Alma 2004; Tedeschi et al. 2008). A better understanding of the interplay between symbionts and immunocytes may explain in part, why some species are vectors and others are not or solve contradictions concerning the vector ability of some species. That is the case of *C. melanoneura,* the main vector of '*Ca.* Phytoplasma mali' in Italy (Tedeschi and Alma 2004), while in Germany it has no relevance as a vector (Mayer et al. 2009).

Moreover, the availability of primary and continuous cell cultures from psyllids will open new opportunities for gene expression studies (Marutanti et al. 2009; Hunter et al. 2009) providing a new tool for screening and discovering novel management chemistries against psyllid phytoplasma vectors. In particular, these cultures aid in current efforts to discover and develop viral pesticides against psyllids.

References

Carraro L, Loi N, Ermacora P (2001) Transmission characteristic of the European stone fruit yellows phytoplasma and its vector *Cacopsylla pruni*. Eur J Plant Pathol 107: 695–700.

Carraro L, Loi N, Ermacora P, Gregoris A, Osler, R (1998) Transmission of pear decline by using naturally infected *Cacopsylla pyri*. Acta Hort 472: 665–668.

Fallon AM, Sun D (2001) Exploration of mosquito immunity using cells in culture. Insect Biochem Mol Biol 31: 263-278.

165 Hodkinson ID (2009) Life cycle variation and adaptation in jumping plant lice (Insecta: Hemiptera:
166 Psylloidea): a global synthesis. J Nat His 43: 65-179.
167 Hunter WB, Dowd SE, Katsar CS, Shatters RG, McKenzie CL, Hall DG (2009) Psyllid biology: expressed
168 genes in adult Asian citrus psyllids, *Diaphorina citri* Kuwayama. The Open Entomology J 3: 18-29.
169 Jarausch B, Fuchs A, Schwind N, Krczal G, Jarausch W (2007) *Cacopsylla picta* as most important vector
170 for '*Candidatus* Phytoplasma mali' in Germany and neighbouring regions. Bull Insectol 60: 189-190.
171 Lavine MD, Strand MR (2002) Insect hemocytes and their role in immunity. Insect Biochem Mol Biol 32:
172 1295-1309.
173 Manfredini F, Dallai R, Ottaviani E (2008) Circulating hemocytes from larvae of the paper wasp *Polistes*
174 *dominulus* (Hymenoptera, Vespidae). Tissue Cell 40: 103–112.
175 Marutani-Hert M, Hunter WB, Hall DG (2009) Establishment of Asian citrus psyllid (*Diaphorina citri*)
176 primary cultures. In Vitro Cell Dev Biol-Animal 45: 317-320.
177 Mayer CJ, Jarausch B, Jarausch W, Jelkmann W, Vilcinskas A, Gross J (2009) *Cacopsylla melanoneura* has
178 no relevance as vector of apple proliferation in Germany. Phytopathology 99: 729–738.
179 Pandey JP, Tiwari RK (2012) An overview of insect hemocyte science and its future application in applied
180 and biomedical fields. Am J Biochem Mol Biol 2: 82-105.
181 Raddadi N, Gonella E, Camerota C, Pizzinat A, Tedeschi R, Crotti E, Mandrioli M, Bianco P, Daffonchio D,
182 Alma A(2011)'*Candidatus* Liberibacter europaeus' sp. nov. that is associated with and transmitted by the
183 psyllid *Cacopsylla pyri* apparently behaves as an endophyte rather than a pathogen. Environ Micro 13:
184 414-426.
185 Smagghe G,Goodman CL, Stanley D (2009) Insect cell culture and applications in research and pest control
186 management. In Vitro Cell Dev Biol-Animal 45: 93-105.
187 Su Q, Zhou X, Zhang Y (2013) Symbiont-mediated functions in insect hosts. Commun Integr Biol 6:
188 e23804.
189 Tedeschi R, Alma A(2004) Transmission of apple proliferation phytoplasma by *Cacopsylla melanoneura*
190 (Homoptera: Psyllidae). J Econ Entomol 97: 8–13.
191 Tedeschi R, Lauterer P, Bertignono L, Alma A(2008) Hawthorn psyllid fauna in northwestern Italy. Bull
192 Insectol 61: 143-144.

Figure legends

Figure 1. Comparison of the growth trends of *C. pyri* in the Sf-900™III (dashed line), Ex-Cell ® 405 (dotted line) and HH70 (continuous line) exemplifying the immunocytes growth observed in psyllid immunocytes cultures. For each medium, datapoints represent a mean of three replicates.

Figure 2. *In vitro* maintained immunocytes of *C. pyri* (a-c), *C. melanoneura* (d-h) and *C. crataegi* (i-n) unstained at light microscopy (a-b, d-f, i-l) and after propidium iodide staining (c, g-h, m-n) can be distinguished in three types: *i*. small cells with the nucleus occupying the central part of the cellular body (b, c, f, g, l, m) (indicated by asterisks); *ii*. large cells with abundant cytoplasm containing cytoplasmic inclusions varying in shape from round to irregular or elongated (a, c, e, h, i, n) (indicated by arrows); *iii*. small cells with a thin cytoplasm layer around a highly defined nucleus (d, g) (indicated by arrow heads). Mitotic cells were frequently observed in *C. pyri* cultures (o-q) after propidium iodide staining. Bars correspond to 10 μm.

Figure 3. Cultured immunocytes of *C. pyri* (a-b, d, e) and *C. melanoneura* (c, f-g) are able to adhere to glass slides losing their spherical shape during adhesion test (a-c) and to phagocytize fluorescent microspheres (d-g) indicating that they are active cells despite their *in vitro* growth.